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PRINCIPAL INVESTIGATOR: Gangyong Li

CONTRACTING ORGANIZATION: Wayne State University
Detroit, Michigan 48201

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6. AUTHOR(S)
Gangyong Li

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Wayne State University School of Medicine
Detroit, Michigan 48201

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13. ABSTRACT *(Maximum 200 words)*

The bcl-2 oncogene product is a potent inhibitor of apoptosis induced by a variety of stimuli. Bcl-2 is highly expressed in breast cancer and its expression is related to chemotherapy and radiation therapy resistance. Increasing evidence suggests that remodeling and turnover of extracellular matrix (ECM) play a critical role in the regulation of apoptosis in mammary epithelial cells. In the present study, our purpose is to investigate whether bcl-2 inhibition of apoptosis involves regulation of ECM-regulating gene expression using three human breast epithelial cell lines: MCF10A, a "normal" breast epithelial cells line; TG3B, a preneoplastic cell line derived from MCF10A; and MCF-7, a breast carcinoma cell line. Overexpression of bcl-2 up-regulates expression of tissue inhibitor of metalloproteinase-1 (TIMP-1), an inhibitor of matrix metalloproteinases (MMPs) in all three cell lines. Bcl-2 down-regulates expression of MMP-9 in MCF10A cells, while MMP-9 was not detectable in TG3B and MCF-7 cells. However, phorbol ester induced MMP-9 expression was abolished by bcl-2 in all three cell lines. We are currently investigating the roles of TIMP-1and MMP-9 in the regulation of apoptosis in breast epithelial cells. Our preliminary result suggests that exogenous TIMP-1 partially protect MCF10A cells against induction of apoptosis by hydrogen peroxide.

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Guangbao Li 6-29-98

PI - Signature Date

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Introduction

Apoptosis, bcl-2 and breast cancer

Apoptosis is a genetically controlled and tightly regulated cellular response to developmental and environmental stimuli culminating in cell death [1,2] It is frequently observed in many physiological and pathological conditions, examples are postlactational involution of mammary gland, embryogenesis, tumor regression and viral infection etc[3]. Apoptosis is important in the regulation of cell population density and inhibition of apoptosis is believed to be one of the mechanisms of unrestrained growth of tumor cells [4,5]. Numerous gene products are involved in the regulation of apoptotic pathways [1]. Bcl-2 protein is one of the molecules capable of suppressing apoptosis induced by a wide variety of stimuli including gamma-irradiation, growth factor withdrawal, chemotherapeutic drugs, neurotrophic factor deprivation, viral infection [6,7]. Bcl-2 is over-expressed in 58-80% of invasive breast cancers and its expression has been related to chemotherapy and radiation therapy resistance [8,9,10].

Extracellular matrix and apoptosis

Extracellular matrix (ECM) is a complex network of macromolecules which are composed of collagens, elastins, proteoglycans, glycoproteins and numerous soluble molecules including growth factors[11]. Turnover and remodeling of ECM is accomplished by matrix metalloproteinases (MMPs), a group of enzymes capable of degrading extracellular matrix. The MMP family includes interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8), stromelysin-1, -2 (MMP-3, -10), stromelysin-3, the 72 kDa type IV collagenase (MMP-2, gelatinase A) and 92 kDa type IV collagenase (MMP-9, gelatinase B)[12,13]. Activities of MMPs are regulated at the levels of transcription, translation, secretion, and activation [13,14]. The MMPs can be inhibited by the tissue inhibitors of metalloproteinases (TIMPs), a conserved family of low molecular weight proteins that includes TIMP-1 (30 kDa)[15], TIMP-2 (21 kDa)[16,17], TIMP-3 (22 kDa) etc[18]. Thus, remodeling and turnover of ECM are regulated by a dynamic balance of activities of MMPs and the levels of TIMPs.

Recent studies suggest that extracellular matrix play a critical role in apoptosis. Overexpression of stromelysin-1 induces apoptosis in mammary epithelial cells in vitro and in transgenic mice [19,20]. Cell death of mammary epithelial cells or small cell lung cancer cells is prevented by the addition of metalloproteinase inhibitor or by culturing those cells on matrix (Englebreth-Holm-Swarm matrix or matrigel) [21,31]. During postlactational involution of the mammary gland in vivo, there is apoptosis of mammary secretory epithelial cells accompanied by proteolytic degradation of the mammary gland basement membrane[22]. It was observed that the levels of sulfated glycoprotein-2 (SGP-2), interleukin-1-beta converting enzyme (ICE), MMP-2, stromelysin-1 and serine proteinase urokinase-type plasminogen activator were all elevated, though some changed earlier and others later [22]. These studies suggest that the degradation of ECM is a relatively common event in the regulation of apoptosis of various types of cells including mammary epithelial cells [23,24]. On the other hand, the extracellular matrix stabilizing proteins such as TIMP-1 and TIMP-2 are shown to protect cells and reduce apoptosis rate (32, 33). However, not all

TIMPs protect cells. TIMP-3 was found to induce cell death in human colon carcinoma cells by stabilizing TNF-α on tumor cell surface(34).

To unveil the mechanism by which bcl-2 inhibits the apoptotic pathway in breast epithelial cells, we transfected MCF10A cells with bcl-2 overexpression vector under the control of the cytomegalovirus promoter (provided by Dr. S. Korsmeyer, Washington University, St.Louis). We have used H_2O_2 and irradiation to induce apoptosis in MCF10A cells. MCF10A cells were chosen for the following reasons: 1. MCF 10A is a spontaneously immortalized cell line which was derived without viral or chemical intervention from mortal diploid human breast epithelial cells [25]. 2. The characteristics of those cells and tissue culture conditions are well established [26]. 3. MCF10A cells contain wild-type p53 gene and undergo apoptotic cell death upon H_2O_2-treatment or irradiation.

Our previous studies show that bcl-2 can down-regulate MMP-9 and up-regulate TIMP-1 in normal breast epithelial MCF10A cells. In order to see whether bcl-2 down-regulation of MMP-9 and up-regulation of TIMP-1 is consistant in preneoplastic or carcinoma cell lines, we included TG3B and MCF-7 cells in our studies. TG3B is the third transplant generation of MCF10AneoT cells which is the MCF10A cells transfected with H-ras gene. TG3B cells represent the preneoplastic breast epithelial cells. MCF-7 cell line is a well-established breast carcinoma cell lines. In order to study bcl-2 effects on MMP-9 and TIMP-1 regulation, we also transfected TG3B cells with the human bcl-2 expressing vector and selected 10 bcl-2 overexpressing clones. We acquired MCF-7 control and bcl-2 overexpressing MCF-7 cells from Dr. Yong Lee's lab (Beaumont Hospital, Royal Oak, MI).

Previous Study Results

Overexpression of bcl-2 inhibits DNA damage-induced apoptotic cell death in MCF10A cells.
The level of bcl-2 overexpression was determined by Western blot analysis and the clones expressing the bcl-2 level three times or higher were chosen (Appendix 1, fig. 1). We have shown that bcl-2 inhibits H_2O_2 induced apoptosis in breast epithelial cells (Appendix 2). Inhibition of apoptosis by bcl-2 was further confirmed by nuclear staining after hydrogen peroxide treatment for 24-48 hours. These results demonstrate that bcl-2 reduces free radical induced apoptosis in breast epithelial cells (Appendix 1)

Bcl-2 down-regulate MMP-9 and up-regulate TIMP-1 proteins
Since extracellular matrix appears to play a critical role in the regulation of apoptosis, we hypothesize that bcl-2 inhibition of apoptosis is related to its ability to modulate the expression of ECM degrading enzymes and/or inhibitors. Initially, we examined the effects of bcl-2 overexpression on the levels of the 72 and 92 kDa type IV collagenases. As shown in Appendix 3, fig. 3, bcl-2 dramatically downregulates expression of 92 kDa type IV collagenase (MMP-9), as determined by gelatin zymograph assay. Since MMP-9 is inhibited by TIMP-1 and TIMP-2, we examined the levels of TIMP-1 and TIMP-2 in the control and bcl-2 overexpressing MCF10A cells. As shown in appendix 4, fig. 3, bcl-2 upregulates the level of TIMP-1 protein, but not TIMP-2 protein, as determined by Western blot analysis. Bcl-2 induction of TIMP-1 was, at least in part, occurs at the mRNA levels, as determined by Northern blot analysis (appendix 4, fig. 4). These

studies suggest that bcl-2 inhibition of apoptosis is mediated by a complex regulation of ECM degrading enzymes and their inhibitors.

Assumption:

A number of studies suggest that remodeling and turnover of ECM play a critical role in the regulation of apoptosis in mammary epithelial cells [19, 20]. Since our preliminary studies show that bcl-2 down-regulates MMP-9, an ECM degrading enzyme, and up-regulates TIMP-1, an inhibitor of MMPs in MCF10A cells, we hypothesize that the death-suppressing activity of bcl-2 in human breast epithelial cells is partly mediated by a complex regulation of matrix-degrading enzymes and their inhibitors. The objectives of our studies are to determine the effects of bcl-2 overexpression on expression of MMPs and TIMPs upon apoptotic stimuli, and to reveal the roles of MMPs and TIMPs in the regulation of apoptosis in human breast epithelial cells. These studies will provide valuable information to unveil the mechanism by which bcl-2 regulates apoptosis in human breast epithelial cells, which may be useful in the design of more rational therapeutic strategies for breast cancer patients.

Body of Report

From June 1997 to May 1998, our research was focused on the objectives one and two. We studied the regulation of MMP-9 and TIMP-1 by bcl-2 at both transcriptional and translational levels. Besides MCF10A cells, We also included two new cell lines, TG3B and MCF-7 in our assay. TG3B cell line is the third transplant generation of MCF10AneoT cells which is H-ras transfected MCF10A cells. We transfected TG3B cells with human bcl-2 expressing vector. Ten bcl-2 overexpressing clones were selected. We obtained MCF-7 control and bcl-2 overexpressing MCF-7 cells from Dr. Yong Lee. In the past year, we also tested the effects of exogenous TIMP-1 on regulation of apoptosis in MCF10A cells with or without hydrogen peroxide treatment.

Experiment Methods

Transfection

One million cells were seeded onto each 100 mm dish. Use 15 μg human bcl-2 expressing vector, 5 μg of hygromycin-resistant vector and 25 μl of lipofectin (GIBCOBRL, Life Technologies, MD) in 5 ml serum-free medium for transfection. Wash the cells with 1xPBS and pour the vector containing medium onto the cell layer. 8 hours later, replace the vector containing medium with complete culture medium plus 100 μg/ml hygromycin (Boehringer Mannheim GMBH, Mannheim, Germany). Pick up the surviving cell colonies and check their bcl-2 levels by Western blot analysis. Cells expressing higher levels of bcl-2 were selected.

Gelatin Zymography

A million cells were seeded onto each 100 mm dish. The next day, the cells were washed with 1xPBS solution and 3 ml of serum-free medium was added to the dish. Next day, the conditioned medium was collected. Equal amount of conditioned medium was mixed with 2xlaemmli non-

reducing sample buffer, incubated for 15 min at room temperature and then electrophoresed on 10% SDS-PAGE gels containing 0.1% gelatin (Novex). After electrophoresis, the gels were washed with 2.5% Triton X-100 twice for 30 min and rinsed 3 times for 30 min with a 50 mM Tris-HCl (PH7.6) buffer containing 5 mM CaCl2, 0.02% Brij-35 (Sigma), 0.2% sodium azide. Then the gels were incubated in this buffer overnight at 37°C. After incubation, the gels were stained with 0.5% Coomassie Brilliant Blue R-250 (Sigma) solution containing 10% acetic acid, 20% methanol for 30 min. The gels were then destained with 7.5% acetic acid solution containing 10% methanol. The matrix-degrading enzymes were detected as clear bands.

Western Blot Analysis

Conditioned medium or whole cell lysates were used. 12% gels were applied in the electrophoresis assay. Samples were mixed 1:1 with 2xSDS sample buffer and boiled for 5 min prior to loading and electrophoretically separated, then transferred to nitrocellulose membranes. After transfer, the membranes were blocked by 5% non-fat milk in 1xTTBS for 1 hour and incubated with first antibodies (Dr. Fridman, Wayne State University, Detroit, MI) for another hour at room temperature. The membranes were washed three times with 1xTTBS solution, then incubated with secondary antibodies (Bio-Rad) for one hour at room temperature. After that, the membranes were washed three times with 1xTTBS solution. The bands were visualized on X-ray films (Kodak, New York) by chemiluminescence agents (PIERCE, Rockford, Illinois).

Northern Blot analysis

Total cellular RNA was isolated using the guanidium-thiocyanate method (6). 10μg of each sample were denatured and separated on a formaldehyde-agarose (1%) gel. The RNA was transferred to a Nytran membrane (Schleicher & Schuell, Keene, NH) using the Turbo blotter (Schleicher & Schuell) in 20xSSC buffer and subsequently UV cross-linked in a stratalinker (Stratagene, La Jolla, CA). Northern blot analysis was carried out by hybridization at 42°C in a solution that contained 50% deionized formamide, 1M NaCl, 50 mM Tris-HCl (PH 7.5), 1mM EDTA, 0.1% SDS, 10xDenhardt's solution, 1 mM NaH2PO4, 1 mM Na2HPO4 and 100μg/ml salmon sperm DNA (GIBCOBRL). The probe for analysis were prepared by random priming.

TPA induction of MMP-9

One million MCF10A,TG3B and MCF-7 cells were seeded onto each 100mm dish. The next day, cells were washed with PBS and 3 ml of serum-free medium with 100 nM TPA (Sigma) was added to each dish. 16 hours later, the conditioned medium was collected from each dish and gelatin zymograph analysis was performed. MMP-9 levels were examined.

Irradiation

Half a million MCF10A cells were seeded into T-25 flasks for overnight growth. The next day, complete medium was replaced with serum-free medium and cells were irradiated at 2 Gy, 4Gy, 6Gy as described in Fig.12. We have been irradiating cells using a 6-MeV linear accelerator at V.A. Hospital in Detroit Medical Center. After 24 hours incubation, the conditioned medium was collected for gelatin zymograph analysis and Western blot analysis.

MMP-9 and TIMP-1 Purification

Purified recombinant human MMP-9 (both active and latent forms) and TIMP-1 proteins are obtained from Dr. Fridman (Wayne State University School of Medicine, MI, a consultant in this

project). MMP-9 was purified as follows: The conditioned medium from Hela cells infected with human MMP-9 vaccinia virus vector was chromatographed in a gelatin sepharose column (Sigma) equilibrated with 1xcollagenase buffer (CB) containing 50mM Tris-HCl (PH7.5), 5mM CaCl2, 150mM NaCl, 0.02% Brij. MMP-9 was eluted with 1xCB and 10% DMSO, then dialyzed against 1xCB overnight. Next, MMP-9 protein was concentrated and protein concentration was determined. Its specificity and activity were determined by Gelatin zymography and Western blot analysis. TIMP-1 purification method was as follows: the conditioned medium of Hela cells infected with human recombinant TIMP-1 vaccinia virus vector was chromatographed in a lectin-Sepharose 4B column (Sigma) equilibrated with a buffer containing 20mM Hepes(pH 7.5), 500 mM NaCl, 1mM CaCl2, 10% glycerol, 0.05% Brij-35 and 0.02% NaN3. TIMP-1 was then eluted with 500 mM methyl a-D-mannopyranoside, diluted in the same buffer. The eluted TIMP-1 was then dialyzed against collagenase buffer, concentrated in a centricon 10 concentrator (Amicon, Beverly, MA) and purified to homogeneity by reverse phase-HPLC. The purity and inhibitory activity of the recombinant TIMP-1 were assessed by silver stain of proteins separated in SDS-PAGE and by inhibition of gelatinase activity, respectively. These experiments were already completed in Dr. Fridman's laboratory.

H_2O_2, exogenous TIMP-1 Treatment

Half a million control and bcl-2 overexpressing MCF10A cells were seeded in 60 mm dishes for overnight growth. The complete medium was replaced with serum free medium. Exogenous TIMP-1 was added . 2-3 hours later, 250 μM hydrogen peroxide was added into the cell medium. 48 hours later, pictures of the cells were taken.

Results and discussion

We have previously shown that bcl-2 inhibit hydrogen peroxide induced apoptosis in MCF10A cells. By carrying out nuclear staining with bisBenzimide (Molecular Probes, Eugene, OR), we proved that cell death caused by hydrogen peroxide treatment is through apoptosis (Appendix 1). When bcl-2 is overexpressed in MCF10A cells, we found that MMP-9 was down-regulated and TIMP-1 was up-regulated in MCF10A cells. Recent findings suggest that extracellular matrix play a critical role in the apoptosis regulation. Overexpression of stromelysin-1, one of the MMP members, induces apoptosis in mammary epithelial cells (19, 20). Overexpressing of TIMP-1, TIMP-2 was shown to inhibit apoptosis (32, 33). Our goal is to understand how bcl-2 regulates MMP-9 and TIMP-1 and the roles of MMP-9 and TIMP-1 in the regulation of apoptosis in our cell systems.

We selected bcl-2 clone #2 and #30 which have high expression of bcl-2 protein (Fig. 1).By repeated experiments, we confirmed that bcl-2 could up-regulate TIMP-1 at both the transcriptional level and translational level in MCF10A cells (Fig. 2). TIMP-1 is a secreted protein and we detected it in the conditioned medium of the cells. MMP-9 is also a secreted protein and it is secreted as a proenzyme. Bcl-2 down-regulation of MMP-9 was proven by both Gelatin zymography and Western blot analysis at the tranlational level (Fig. 3, 4). However, Our Northern blot analysis failed to detect MMP-9 mRNA in both MCF10A control and bcl-2 overexpressing MCF10A cells. The reason might be that MMP-9 mRNA level is not high enough for Northern blot analysis detection, although MMP-9 mRNA level in control cells may be higher than that of bcl-2

overexpressing MCF10A cells. Gelatin zymograph analysis is sensitive enough to detect MMP-9 in the conditioned medium. MMP-2 is also detectable, it seems that MMP-2 levels in bcl-2 over-expressing cells are slightly higher than that of control cells, but the elevation is not significant enough to make a statement for this cell line.

To test whether bcl-2 regulation of MMP-9 and TIMP-1 are common effects among breast epithelial cells, we included TG3B cells and MCF-7 cells in our assay. We established bcl-2 over-expression clones by transfecting TG3B cells with human bcl-2 expressing vector and their bcl-2 levels were determined by Western blot analysis (Fig. 5). We also obtained MCF-7 control and several bcl-2 overexpressing MCF-7 cells from Dr. Yong Lee's lab (Fig. 7). By carrying out both Western blot analysis and Northern blot analysis, we found that TIMP-1 mRNA and protein levels were all elevated in bcl-2 overexpressing TG3B and MCF-7 cells compared with their control cells, indicating that bcl-2 up-regulation of TIMP-1 mRNA and protein is common effect of bcl-2 over-expression among breast epithelial MCF10A cells (Fig. 2), TG3B cells (Fig. 6) and MCF-7 cells (Fig. 8). For TG3B and MCF-7 cell lines, the MMP-9 basal levels were barely detectable in both parental and bcl-2 over-expressing cells. In order to see bcl-2 effect on MMP-9 expression, we used a potent MMP-9 inducer, Phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), to treat TG3B and MCF-7 cells. It was found that TPA was able to induce MMP-9 in TG3B, MCF-7 and MCF10A parental cells but not in bcl-2 overexpressing TG3B , MCF-7 and MCF10A cells, indicating that bcl-2 could inhibit MMP-9 expression in all three cell lines (Fig. 9 , 10, 11). We are currently investigating the role of MMP-9 on apoptosis.

Irradiation and hydrogen peroxide treatment was frequently used to induce apoptosis in our experiment. We tested whether irradiation and hydrogen peroxide treatment can alter MMP-9 and TIMP-1 expression in MCF10A cells. In our irradiation test, MMP-9 levels did not change. TIMP-1 levels increased and they increased in a dose dependent manner (Fig. 12). Hydrogen peroxide did not affect MMP-9 and TIMP-1 expression. We are investigating the effect of radiation-induced TIMP-1 on radiation-resistance and radiation-induced apoptosis.

We tested how exogenous TIMP-1 affect apoptosis rate in MCF10A cells. Since bcl-2 overexpressing MCF10A cells secreted more TIMP-1 than parental MCF10A cells, we tested whether TIMP-1 is responsible for the protection against apoptosis. So we added TIMP-1 protein to parental MCF10A cells. Two hours later, we added 250 μM of hydrogen peroxide to induce cell death. 24 and 48 hours later, we examined the cells and found that in MCF10A cells with exogenous TIMP-1, there was less cell death as compared with MCF10A cells without adding TIMP-1 protein (Fig. 13). To examine the roles of TIMP-1 in apoptosis, we have estabalished TIMP-1 over-expressing MCF10A clones. We are currently characterizing these new TIMP-1 over-expressing cells.

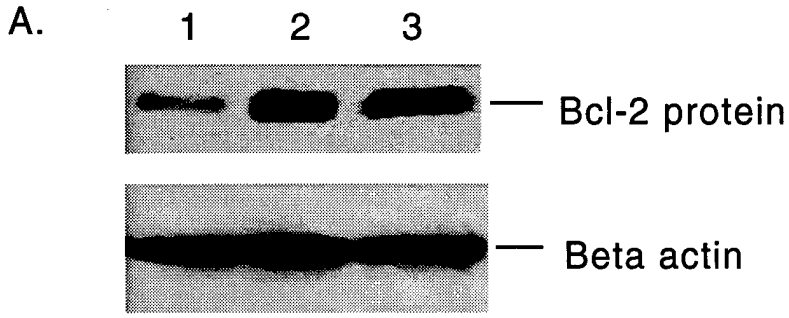

Figure 1. Western blot analysis of bcl-2 protein from MCF10A control (lane 1) and bcl-2 overexpressing MCF10A cells (lanes 2 and 3). Lanes 2 and lane 3 represent bcl-2 overexpressing clone #2 and#30. The lower panel shows the protein loading of the same blot.

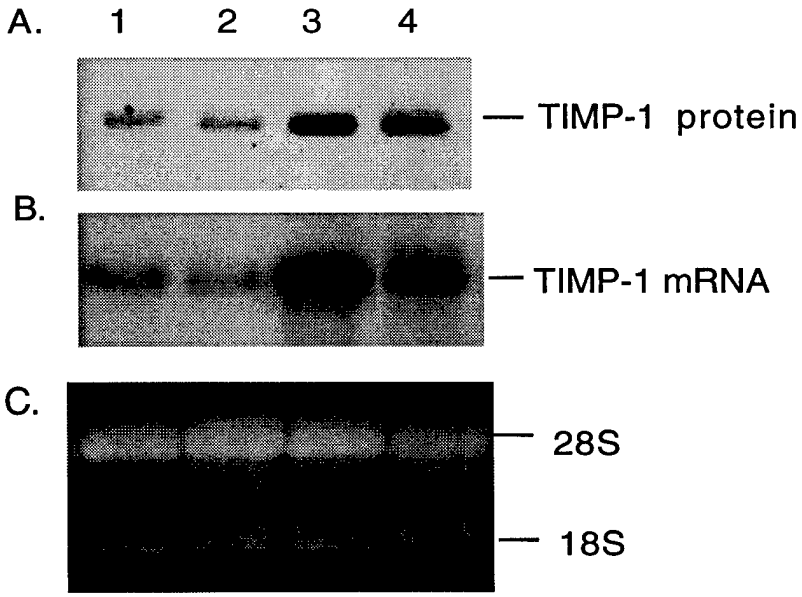

Figure 2. Panel A. Western blot analysis of TIMP-1 from conditioned media of MCF10A control cells (lanes 1 and 2) and bcl-2 overexpressing cells (lanes 3 and 4). Lane 3 and 4 represent bcl-2 clones #2 and # 30 respectively. Panel B. Northen blot analysis of TIMP-1 mRNA from cell lysates of MCF10A controls and bcl-2 overexpressing cells. The lanes are the same as in panel A. Panel C. shows the RNA loading of Panel B.

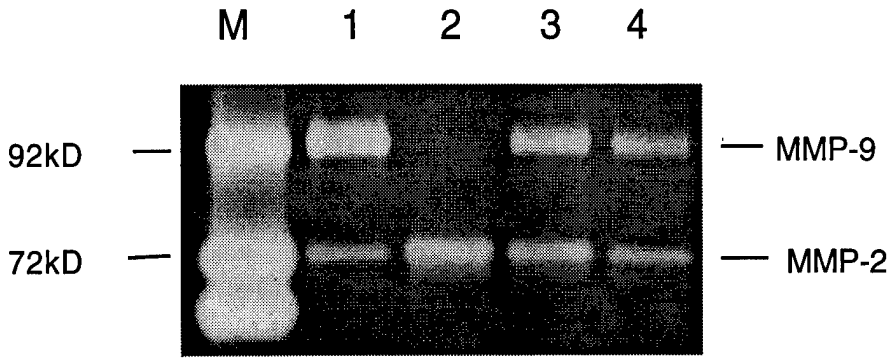

Figure 3. Gelatin zymography of MCF10A control cells(lane 1) and bcl-2 overexpressing MCF10A cells (lanes 2, 3, 4 represents bcl-2 clones #2, #30 and pooled population). The upper bands indicate MMP-9 which is a 92kDa type IV collagenase. The lower bands indicat MMP-2 which is a 72 kDa type IV collagenenase.

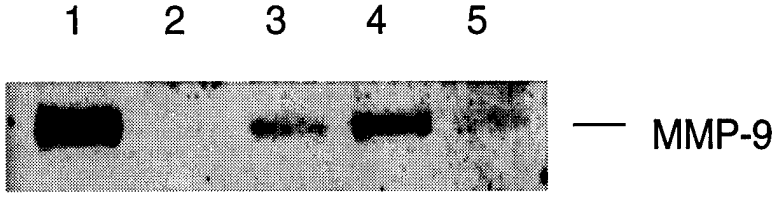

Figure 4. Western blot analysis of MMP-9 (92 kDa type IV collagenase) from conditioned media of MCF10A control (lane 1) and bcl-2 overexpressing MCF10A cells (lane 2, 3, 4 and 5). Lanes 2, 3, 4 and 5 represent bcl-2 overexpressing clones #2, #30, #40 and pooled populations respectively. Equal amount of protein was loaded.

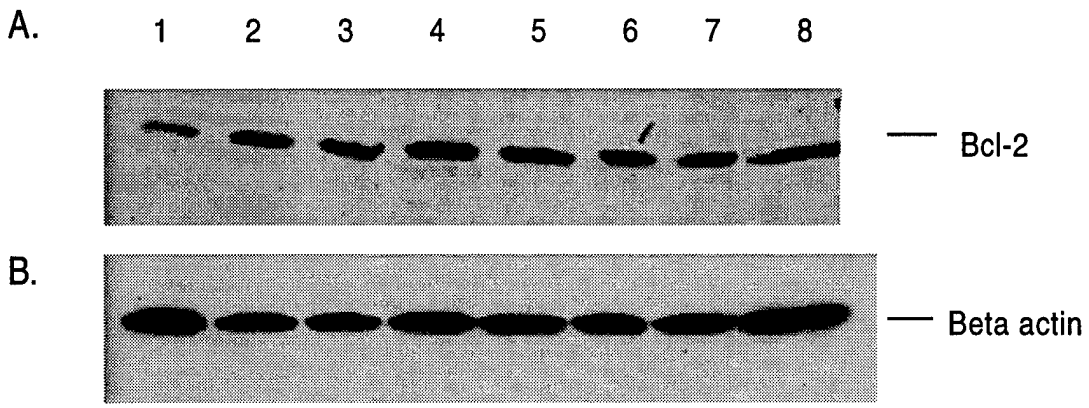

Figure 5. Panel A. Western blot analysis of bcl-2 protein from cell lysates of TG3B control cells (lane 1) and bcl-2 overexpressing TG3B cells(lanes2 to 8). Panel B. To confirm the amount of protein loading, the blot was probed with anti-beta actin antibody.

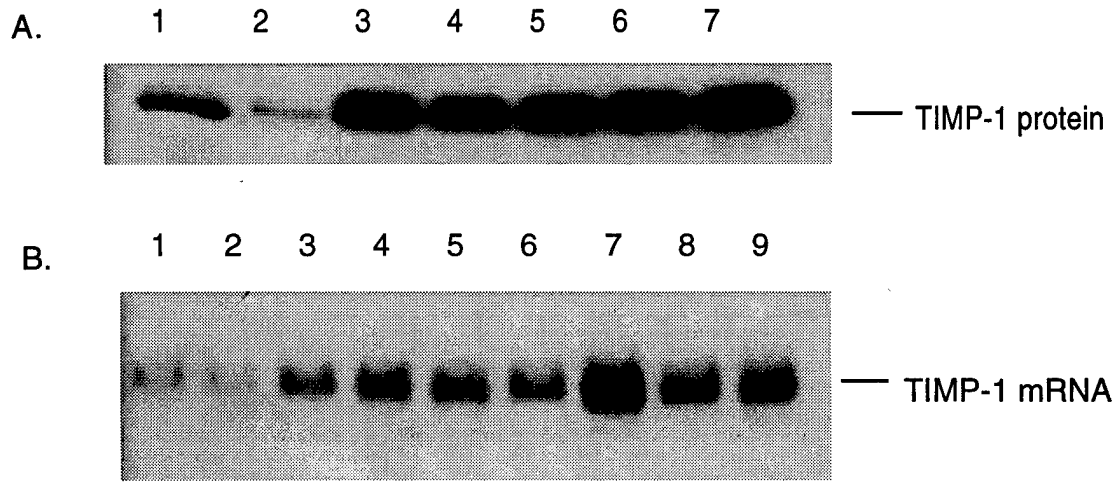

Figure 6. Panel A. Western blot analysis of TIMP-1 protein from conditioned media of TG3B and bcl-2 transfected TG3B cells. Lane 1 and 2 are TG3B control cells. Lanes 3 to 7 are bcl-2 overexpressing TG3B cells. Panel B. Northern blot analysis of TIMP-1 mRNA from the cell lysates of TG3B control (lanes 1 and 2) and bcl-2 overexpressing TG3B cells (lanes 3 to 9).

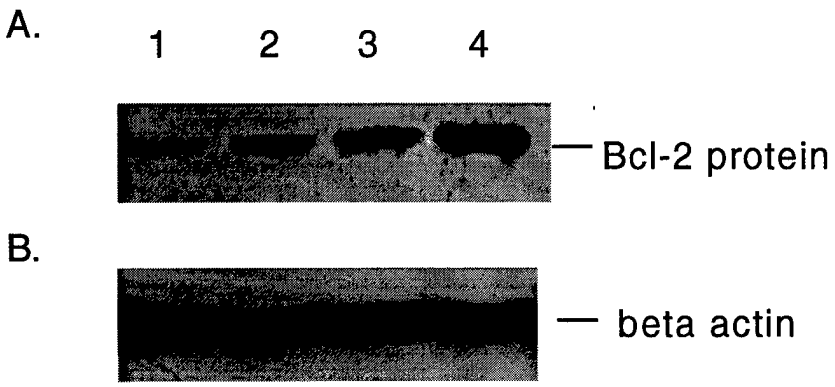

Figure 7. Panel A. Western blot analysis of bcl-2 proteins from MCF-7control (lane 1) and bcl-2 overexpressing MCF-7 cells (lanes 2, 3, and 4 represents bcl-2 overexpressing clones #4, 5, and 6 respectively). Panel B shows the protein loading of panel A.

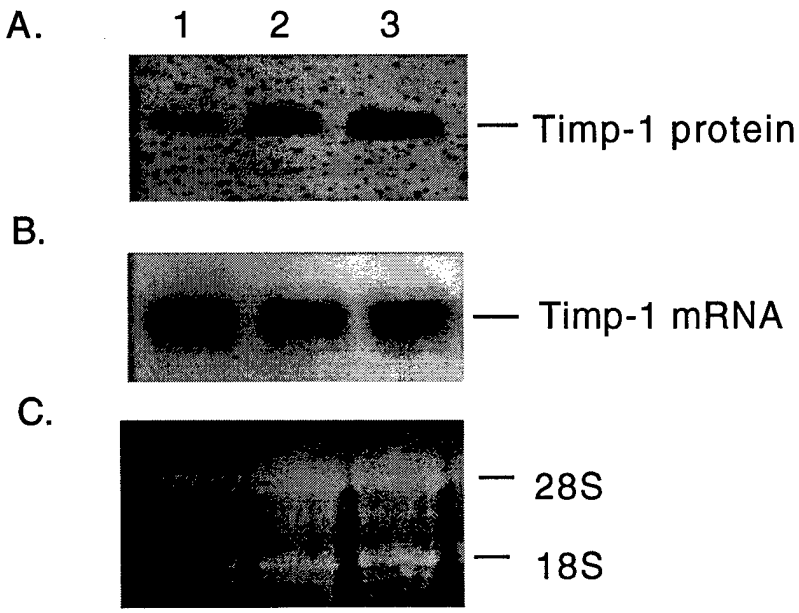

Figure 8. In panels A, B, and C, lane 1 is MCF-7 control cells. Lanes 2 and 3 represent bcl-2 overexpressing MCF-7 clones #5 and #6 respectively. Panel A. Western blot analysis of secreted Timp-1 protein from conditioned media of MCF-7 control cells and bcl-2 overexpressing MCF-7 cells. Panel B. Northern blot analysis of Timp-1 mRNA from cell lysates of MCF-7 control and bcl-2 overexpressing cells. Panel C shows the RNA loading of Northern blot analysis in panel B.

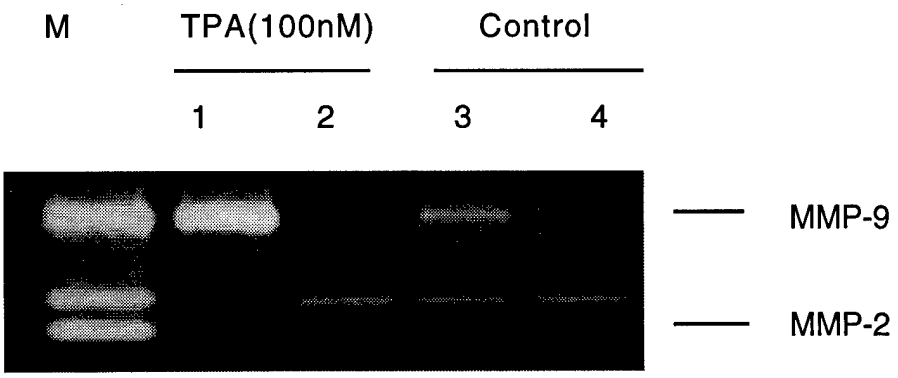

Figure 9. Gelatin zymography of MCF10A and bcl-2 overexpressing MCF10A cells. M indicates enzyme markers. Lanes 1 and 2 are MCF10A and bcl-2 overexpressing MCF10 cells treated with 100nM TPA for 24 hours respectively. Lanes 3 and 4 are MCF10A and bcl-2 overexpressing MCF10A respectively . They serve as controls.

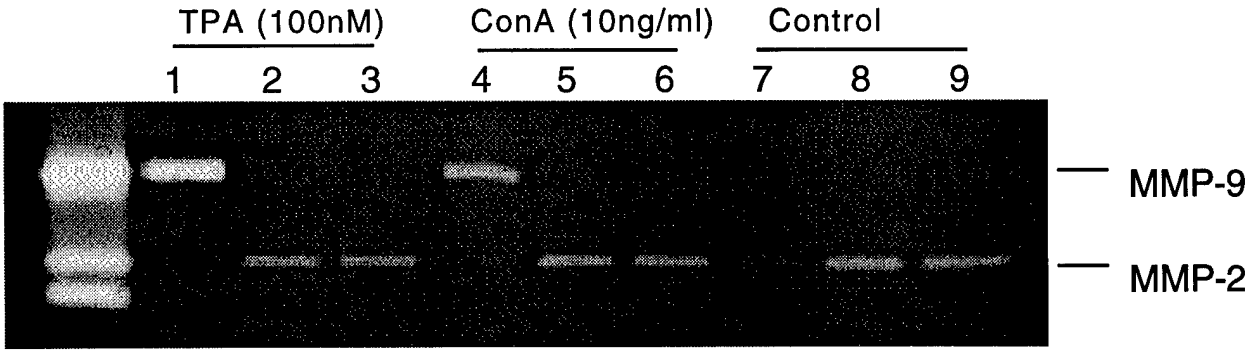

Figure 10. Gelatin zymography of TG3B control and bcl-2 overexpressing TG3B cells. Lanes 1, 4 and 7 are the seme control cells. Lane 2, 5 and 8 are bcl-2 overexpressing clone #1. Lanes 3, 6 and 9 represent bcl-2 overexpressing clone #3. Lanes 1,2, and 3 are treated with 100nM of TPA for 24 hours. Lanes 4, 5 and 6 are treated with 10ng/ml ConA. Lanes 7, 8 and 9 are controls without any treatment.

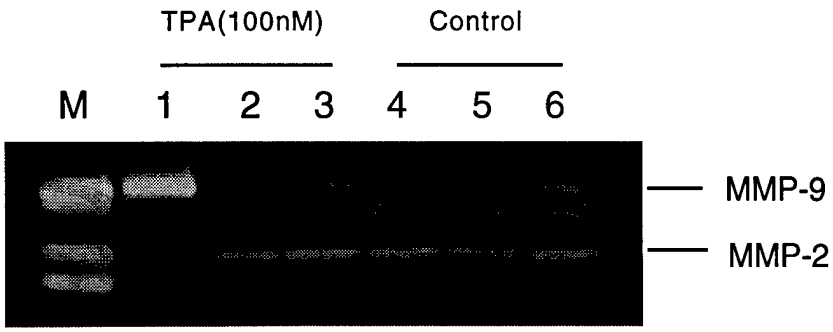

Figure11. Gelatin zymography of MCF-7 and bcl-2 over-expressing MCF-7 cells. M indicates enzyme marker. Lanes 1 and 4 are MCF-7 control cells. Lanes 2 and 5 represent MCF-7 bcl-2 over-expressing clone #5. Lanes 3 and 6 represent bcl-2 over-expressing clone#6. . As indicated above, lanes 1, 2 and 3 are with TPA treatment. Lanes 4, 5 and 6 are controls without TPA treatment.

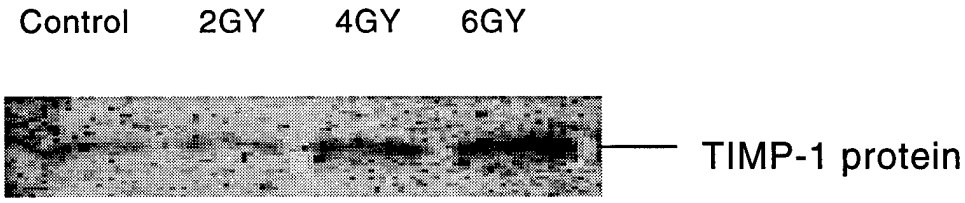

Figure 12. Western blot analysis of TIMP-1 protein from conditioned Medium of MCF10A cells after irradiation. 2GY, 4GY and 6 GY indicate different doses of irradiation.

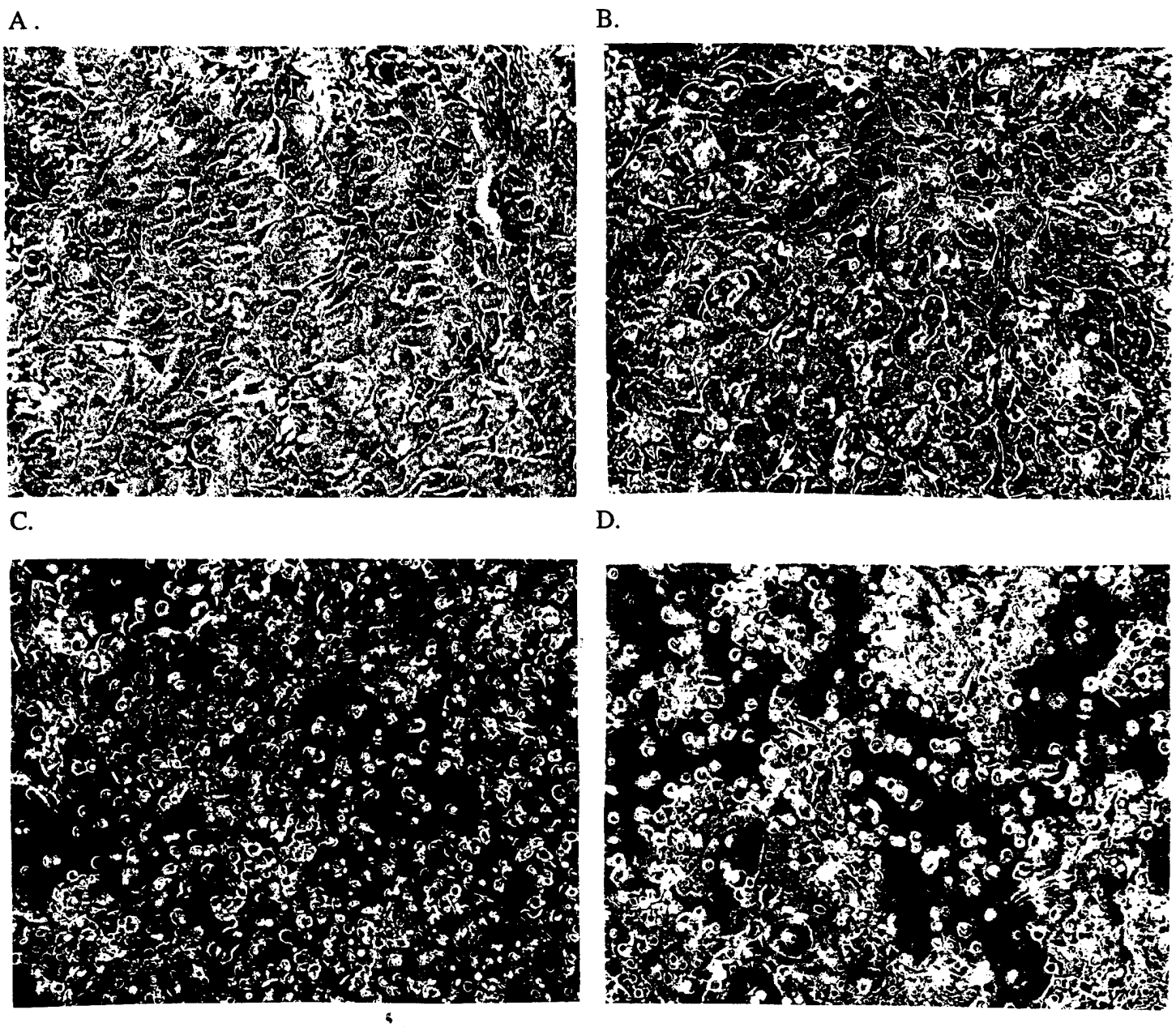

Figure 13. Treatment of MCF10A cells with TIMP-1 and hydrogen peroxide. Half a million cells were seed onto each 60 mm dish. The next day, cells were washed with PBS twice and different serum-free media were added into the cells.

- Picture A. Only serum-free medium was added.
- Picture B. Serum-free medium and exogenous TIMP-1 (1μg/ml) were added.
- Picture C. Serum-free medium, TIMP-1(1μg/ml) were added first, two hours later, hydrogen peroxide (250μM) was added into the dishes.
- Picture D. Serum-free medium and hydrogen peroxide were added into the dishes.
- 24 hours later, cells were examined. 48 hours later, pictures were taken.

Conclusion

MCF10A, TG3B and MCF-7 are the three breast epithelial cell lines used in our experiment. MCF10A represents normal breast epithelial cells. TG3B represents preneoplastic breast epithelial cells. MCF-7 is the breast carcinoma cell line. Our purpose is to understand whether bcl-2 inhibition of apoptosis is partially through remodeling and turnover of extracellular matrix components. These studies will provide valuable information to unveil the mechanism by which bcl-2 regulates apoptosis in human breast epithelial cells, which may be useful in the design of more rational therapeutic strategies for breast cancer patients.

In our study, we found that:

1. Bcl-2 overexpressing cells are generally more resistant to apoptotic signals such as irradiation and hydrogen peroxide treatment, indicating bcl-2 as a potent inhibitor of apoptosis.

2. Bcl-2 up-regulates TIMP-1 mRNA and TIMP-1 protein in all three breast cell lines. The results imply that bcl-2 may exert its anti-apoptotic activity through up-regulation of ECM stabilizing proteins such as TIMP-1.

3. In MCF10A cells, bcl-2 down-regulates basal level of MMP-9 expression, while MMP-9 expression was not detectable in TG3B and MCF-7 cells.

4. Bcl-2 inhibits the induction of MMP-9 expression by Phorbol ester TPA in all three cell lines, implying that MMP-9 may be involved in apoptosis pathway.

5. Irradiation induces TIMP-1 expression in a dose-dependent manner in MCF10A cells. Hydrogen peroxide treatment did not alter either MMP-9 or TIMP-1 expression in MCF10A cells.

6. Exogenously added TIMP-1 partially protects MCF10A control cells against induction of apoptosis. The result suggests that TIMP-1 is involved in apoptosis pathway. More experiment needs to be done in this area to confirm the role of TIMP-1 in regulation of apoptosis.

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Appendix

A. Acronym and Symbol Definition: described in the text.

B. Illustrations:

Figure 1.

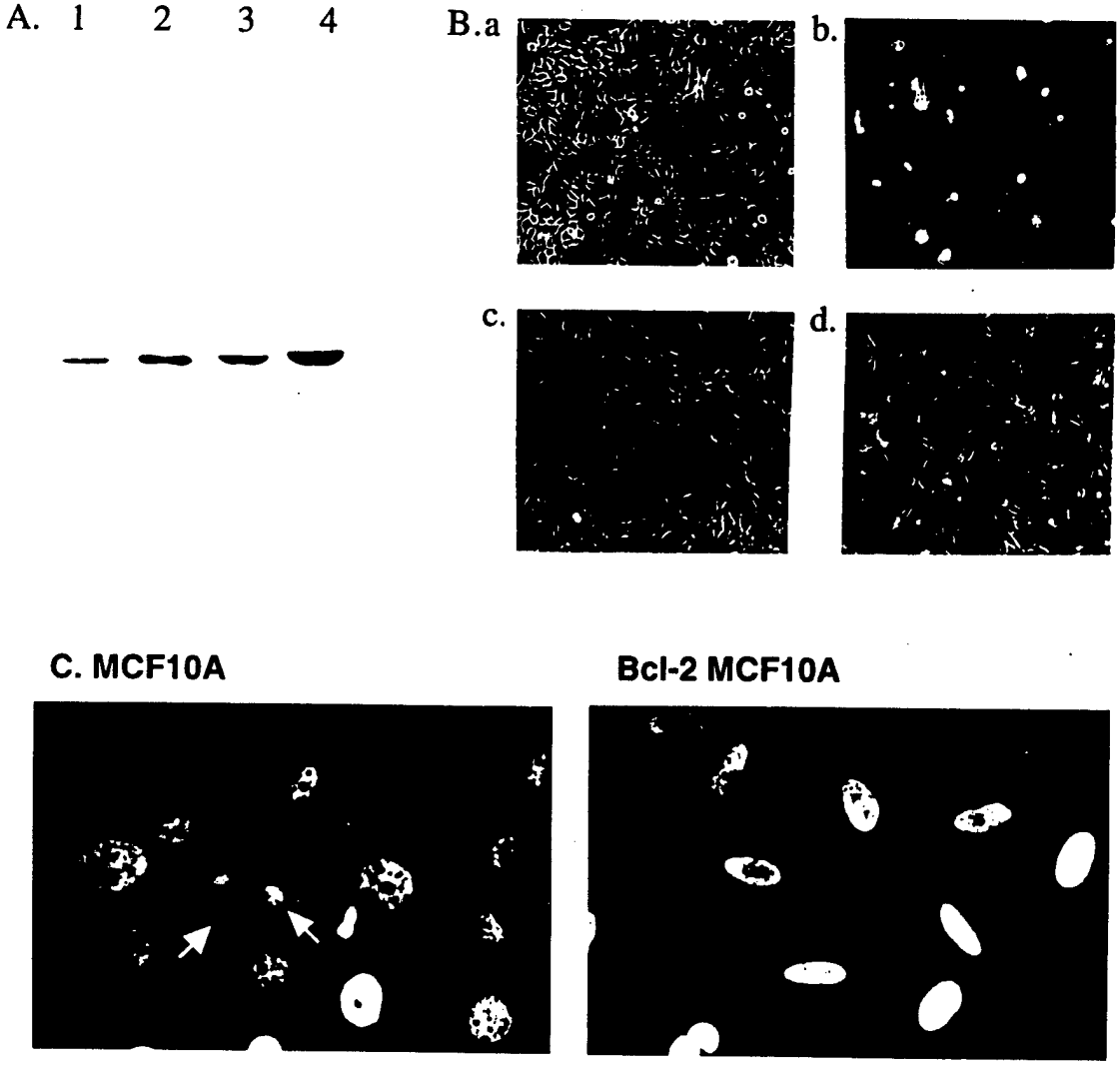

Figure 1. Characterization of bcl-2 overexpressing MCF10A cells.
A. Western blot analysis of bcl-2 protein.The total protein from control (lane 1) and bcl-2 expressing MCF10A clones (lane 2, clone 2; lane 3, clone 10; lane 4, clone 30) were extracted. The levels of bcl-2 protein were determined using anti-bcl-2 antibody.
B. $5x10^5$ control MCF10A (a and b) and bcl-2 expressing MCF10A(c and d) cells were plated in 6-well plates. After overnight culture, 500 μM hydrogen peroxide was added to the control (b) and bcl-2 expressing cells (d) for 48 h.
C. Nuclear morphologies of the control and bcl-2 expressing cells (clone 2) after 24 h of treatment with 500μM hydrogen peroxide were examined using bisBenzimide. Arrows point to fragmented nucleus of apoptotic cells.

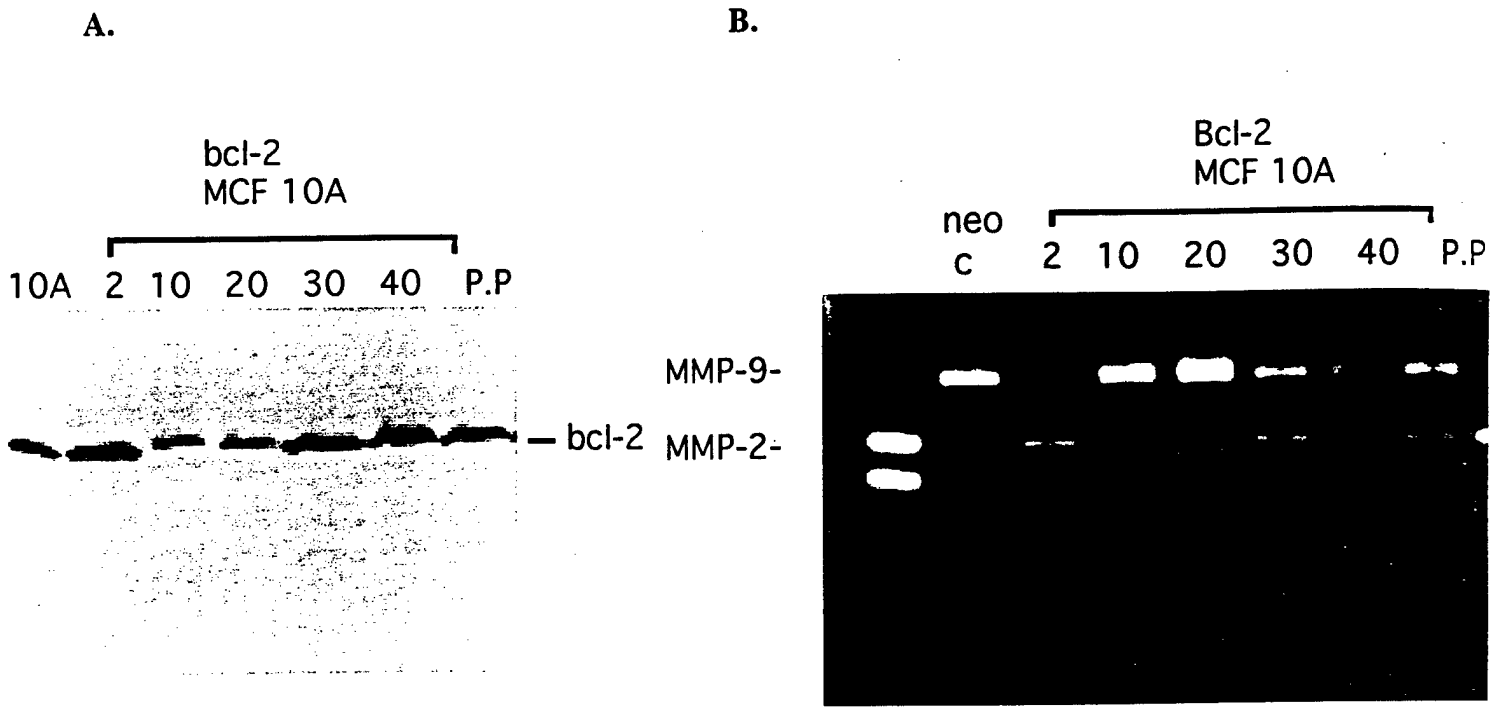

Figure 2. Overexpression of bcl-2 downregulates expression of 92-kDa collagenase in human breast epithelial cells.

A. Western blot analysis of bcl-2 protein in control MCF10A cells and bcl-2 overexpressing MCF10A clones (clone 2, 10, 20, 30, 40 and pooled population). 10μg of total cell lysate was electrophoresed on 16% SDS-polyacrylamide gel, electrophoretically blotted to nitrocellulose membrane and probed with anti-bcl2 antibody .

B. Gelatin Zymograph Assay: Recombinant human MMP-2 control (lane1). Conditioned media from control MCF10Aneo cells (lane 2), bcl-2 overexpressing MCF10A clones 2 (lane 3), 10 (lane 4), 20 (lane 5), 30 (lane 6), 40 (lane 7) and pooled population (lane 8) were electrophoresed on 10% SDS-polyacrylamide gel containing 0.1% gelatin. The clear bands indicate enzymatic digestion of gelatin.

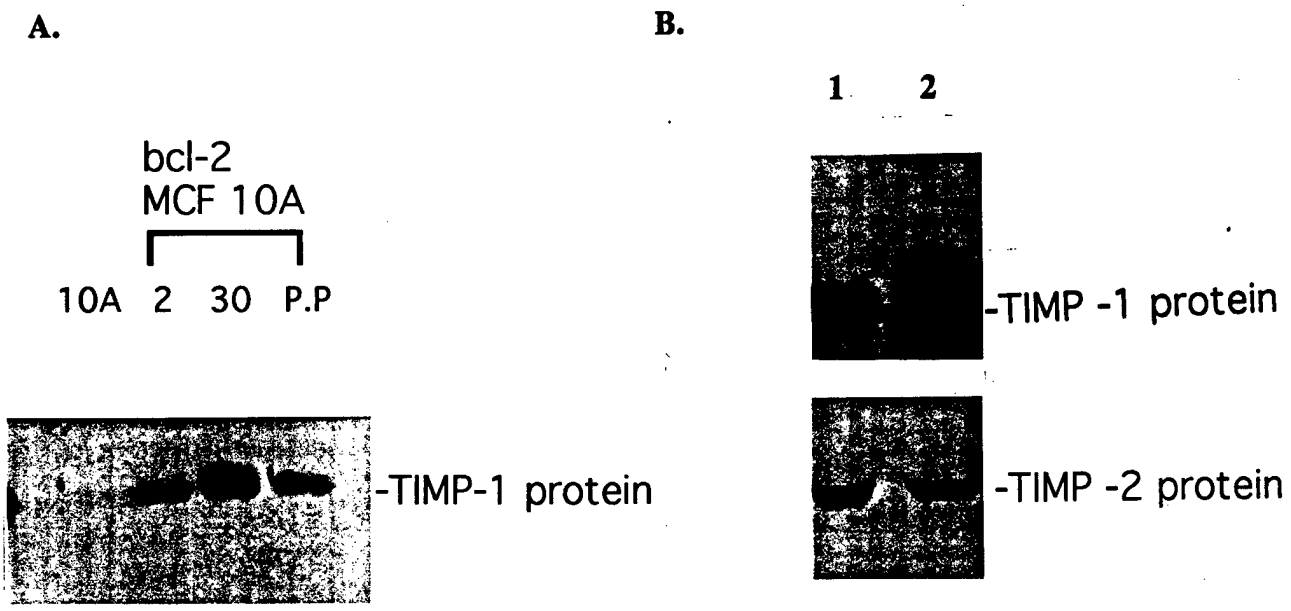

Figure 3. Bcl-2 upregulates the level of TIMP-1 protein, but not TIMP-2 protein.
A. Western blot analysis of Secreted TIMP-1. Conditioned media from MCF10A (lane 1), bcl-2 overexpressing MCF10A clone 2 (lane 2), clone 30 (lane 3), pooled population (lane 4), were electrophoresed on a 16% SDS-polyacrylamide gel, blotted to nitrocellulose membrane and probed with anti-TIMP-1 antibody.
B. Wsetern blot analysis of TIMP-1 and TIMP-2 proteins. Proteins from MCF10A cells (lane 1), MCF10Abcl2-2 (lane 2), were electrophoresed on a 16% SDS-polyacrylamide gel, blotted to a nitrocellulose membrane and probe with anti-TIMP-1 antibody (top panel) or anti-TIMP-2 antibody (bottom pannel).

Figure 4.

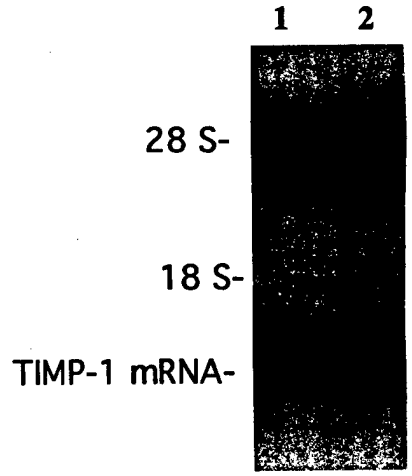

Figure 4. Bcl-2 upregulates the level of TIMP-1 RNA.
Northern blot analysis of TIMP-1. 5μg of total RNA were run on a 1% agarose gel containing 0.66M formaldehyde, transfered to a nytran membrane, probed with ^{32}p-labeled human TIMP-1 cDNA probe. Lane 1 is MCF10Aneo control cells. Lane 2 is MCF10Abcl2-2.

Figure 5.

Days:	1	2	3
MCF10A:	100%	39%	19%
MCF10Abcl2-2:	100%	88%	52%
MCF10Abcl2-30:	100%	68%	72%

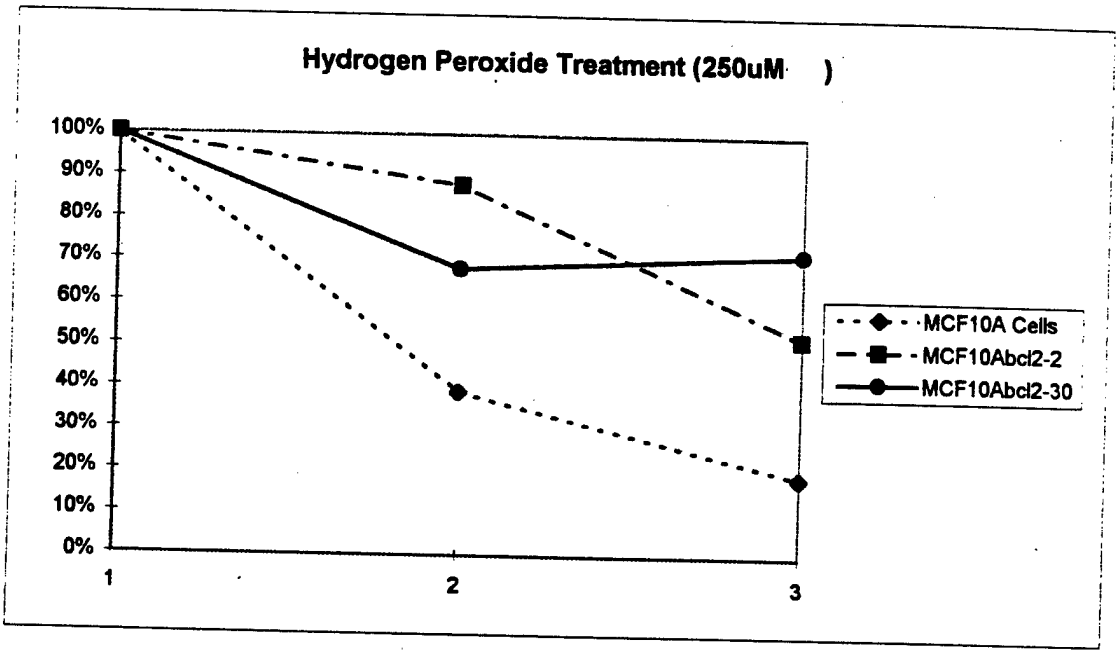

Figure 5. Hydrogen Peroxide induced cell death of control and bcl-2 Overexpressing MCF10 Cells

$5x10^5$ MCF10A and bcl-2 overexpressing MCF10 cells are seeded into 60mm dish. The next day, culture medium is replaced with serum-free medium. 250 μM H_2O_2 is added into the medium of the cells. Each cell line has its own control cells which are cultured in serum free medium without adding H_2O_2. 24 or 48 hour later, control and H_2O_2 treated cells are counted. The number of control cells is set to be 100%. The ratios of surviving cells of the H_2O_2-treated cells to their own controls are calculated respectively.